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Comparative studies on physiology and taxonomy f obligately purinolytic clostridia

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Abstract. Eleven strains of obligately purinolytic clostridia have been studied with respect to their assignment to the three type strains of *Clostridium acidiurici*, *C. cylindrosporum*, and *C. purinolyticum*. DNA/DNA-hybridization proved to be the method of choice for differentiation whereas phenotypic characteristics such as spore morphology, substrate spectra, nutritional requirements, product formation, and sensitivity against various antibiotics did not allow unequivocal identification. All strains depended on selenite for growth.

Key words: *Clostridium acidiurici* — *Clostridium cylindrosporum* — *Clostridium purinolyticum* — Purine metabolism — Selenite — Antibiogram — DNA homology — Taxonomy

The first report on a strictly anaerobic, sporeforming purinolytic bacterium was already given at the beginning of this century by Liebert (1909). He described *Bacillus acidiurici*, an organism that fermented uric acid to acetate, CO_2, and NH_3. His studies were continued by Barker and Beck (1941, 1942) who isolated and characterized *Clostridium acidiurici* [highly probable Liebert's *B. acidiurici* (Vogels and van der Drift 1976)] and *C. cylindrosporum*. These two organisms only used purines such as uric acid, xanthine, hypoxanthine, and guanine, but not adenine, as growth substrates (Barker and Beck 1942; Beck 1948). Besides morphological differences such as spore form and spore location *C. cylindrosporum* could be separated by its production of glycine and formate besides acetate (Barker and Beck 1941, 1942). For more than 30 years this was the last taxonomic study on obligately purine-fermenting clostridia. In 1977 Champion and Rabinowitz isolated 9 new strains of strictly anaerobic, purinolytic sporeformers and classified them as *C. acidiurici* and *C. cylindrosporum*, respectively, on the basis of formate formation and chemical and immunochemical data on the two proteins ferredoxin and formyltetrahydrofolate synthetase. These criteria did not yield clear-cut results in all cases since one of the strains, AAM-1, pr ved to be quite atypical. The ambiguity was resolved by the identification of a new species of obligately purinolytic clostridia, *C. purinolyticum* (Dürre et al. 1981). This organism was able to ferment adenine and glycine in addition to the afore-mentioned purine derivatives and strictly depended on selenium compounds for growth. Strain AAM-1 turned out to be a subspecies of *C. purinolyticum* (Dürre et al. 1981). Since tests for utilization of adenine and glycine and dependence on selenium compounds are easy to perform, the identification of strains of this species seems to be no problem at all. In contrast, the differentiation between *C. acidiurici* and *C. cylindrosporum* has only been achieved by means of laborious and time-consuming methods such as DNA/DNA-hybridization (Dürre et al. 1981), determination of trace element requirements for the enzyme formate dehydrogenase (Wagner and Andreesen 1977), and immunochemical investigations (Champion and Rabinowitz 1977).

This paper compares the physiological characteristics of all new strains of obligately purinolytic clostridia, and gives the respective data of DNA/DNA-hybridization studies. On the basis of these results it is proposed to maintain *C. cylindrosporum* as a separate species, and a phylogenetic relationship of these organisms is suggested.

Materials and methods

Clostridium acidiurici 9a (DSM 604), *C. cylindrosporum* HC-1 (DSM 605), and *C. purinolyticum* WA-1 (DSM 1384) were obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany. Strains AAM-1, AAM-2, AC-1, AC-3, MBJ-2, MJ-2, MJ-6, NOA-1, and NOA-2 were kindly provided by J.C. Rabinowitz, University of California, Berkeley, USA. Strain IMS was isolated by students from sludge of the sewage plant in Göttingen, FRG. PD-1 was from our laboratory collection. *C. sticklandii* (DSM 519) was kindly provided by A.C. Schwartz, University of Bonn, FRG. Composition and preparation of growth media have been published previously (Dürre et al. 1981). Variations used are described in the experimental section.

Analyses were carried out as follows: purine derivatives were determined spectrophotometrically at wavelengths of their respective ultraviolet absorbance maxima or by the high-pressure liquid chromatographic method of Dürre and Andreesen (1982a), acetate was measured by an enzymatic procedure (Dorn et al. 1978), formate by the colorimetric method of Lang and Lang (1972), and glycine as described by Sardesai and Provido (1970). Turbidity was determined at 600 nm in a Bausch and Lomb Spectronic 88 or a Zeiss PM 4 spectrophotometer, respectively.

Preparation of cell-free extracts and the assay systems for formate dehydrogenase and xanthine dehydrogenase have been reported earlier (Dürre and Andreesen 1983; Wagner

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Table 1. Morphology and spore form of purinolytic clostridia

Strain	Gram stain	Average length (μm)	Diameter (μm)	Spore form	Spore location
Clostridium acidiurici 9a	+	3.9	0.6	oval	terminal, slight swelling of mother cell
C. cylindrosporum HC-1	−/+	3.3	0.8	cylindrical	terminal to subterminal
C. purinolyticum WA-1[a]	+	6.2	1.4	spherical	terminal, marked swelling of mother cell
AAM-1	+	3.3	0.6	cylindrical	terminal to subterminal
AAM-2	+	2.6	0.7	oval to cylindrical	terminal
AC-1	+	1.9	0.6	oval	terminal, slight swelling of mother cell
AC-3	+	5.9	0.7	oval	terminal
IMS	−/(+)	2.9	0.6	oval	terminal
MBJ-2	+	2.6	0.8	oval	terminal to subterminal
MJ-2	+	3.9	0.8	oval	terminal
MJ-6	+	1.9	0.6	cylindrical	terminal to subterminal
NOA-1	+	2.6	0.8	oval	terminal, slight swelling of mother cell
NOA-2	+	2.6	0.7	oval to cylindrical	terminal, to subterminal, slight swelling of mother cell
PD-1	+	4.3	0.8	cylindrical	subterminal

[a] Data were taken for comparison from Dürre et al. (1981)

and Andreesen 1979). Protein determination of extracts was performed according to Beisenherz et al. (1953). Substrate utilization was tested as already described (Dürre et al. 1981).

Isolation of DNA and estimation of G + C content (mol percent guanine plus cytosine) by the thermal denaturation method were performed by a slight modification of the procedures of Marmur and Doty (1962) as described by Auling et al. (1980). DNA/DNA-reassociation experiments were carried out according to De Ley et al. (1970) using a Gilford spectrophotometer 250 equipped with a thermoprogrammer 2527.

Decoyinine was a gift from P. Fortnagel, Institute of Microbiology, Hamburg, FRG. Antibiotics were from Becton Dickinson GmbH, Heidelberg, FRG. 8-Hydroxypurine was synthesized according to Isay (1906). All other chemicals used were of the highest purity commercially available.

Results

Morphology

All strains grew in liquid medium as single rods. Sometimes chains of 2–4 cells could be observed. Average cell lengths and diameters are listed in Table 1. Spores were hardly found in liquid cultures. Addition of decoyinine, an inhibitor of purine nucleotide synthesis that highly induces sporulation in bacilli (Mitani et al. 1977), at a concentration of 1.8 mM showed no increase of the sporulation rate of the clostridia. Spore form was oval to cylindrical with terminal to subterminal location (Table 1). Swelling of mother cells could only be observed in a few cases.

With the exceptions of strain IMS and *Clostridium cylindrosporum* HC-1 that were Gram-negative or variable, all strains stained Gram-positive when young and actively growing cultures were analyzed.

Optimization of growth conditions

All strains were able to grow in a temperature range between 19°C and 45°C. Higher temperatures proved to be inhibitory. The optimal values as determined by the growth rate are given in Table 2. The influence of the starting pH on growth rates was determined with uric acid and hypoxanthine as a substrate, respectively (Table 2). The final pH was about 9 regardless of the strain used. Yeast extract as a supplement was necessary for some strains. With the exception of *C. purinolyticum* WA-1 that needed about 0.01% (w/v) yeast extract for growth, all other cultures either required only 0.003% of complex nutrients or nothing at all (Table 2). In all cases the requirement for yeast extract could be supplemented for by the vitamins thiamine and biotin. *C. purinolyticum* WA-1, AAM-1, AC-3, MBJ-2, and NOA-2 only needed 0.15 μM thiamine while *C. cylindrosporum* HC-1 required 0.15 μM thiamine and 40 nM biotin (Table 2). With uric acid as the substrate a supplementation with 100 mM potassium bicarbonate proved to be stimulating only for AAM-2. However, addition of bicarbonate was essential for growth on hypoxanthine at a starting pH of 7.2 (Table 3). After increasing the pH to 8.0 AAM-1, MJ-6, and NOA-2 were able to grow without bicarbonate, while the concentration necessary for reaching the maximal turbidity proved to be 30 mM. Optimal concentration of $KHCO_3$-supplementation were 60 mM for *C. acidiurici* 9a, AAM-2, and MBJ-2 and 100 mM for *C. cylindrosporum* HC-1, MJ-2, and NOA-1. AC-3 could not grow with hypoxanthine at all.

The use of various reducing agents such as thioglycolate, sulfide, dithionite, or cysteine for medium preparation did not have an effect on growth of all strains with the only exception being cysteine. This substance decreased the maximal optical density reached in stationary phase down to 15%.

Table 2. Optimal growth parameters for purinolytic clostridia

Strain	Temperature (°C)	Yeast extract necessary for growth %(w/v)	Vitamin requirements	pH Optimum with	
				Uric acid	Hypoxanthine
C. acidiurici 9a	40–45	0	–	6.7	8.0
C. cylindrosporum HC-1	40–45	0.003	thiamine, biotin	7.5	8.0
C. purinolyticum WA-1[a]	36	0.01	thiamine	n.d.	7.3–7.8[b]
AAM-1	36	0.003	thiamine	7.5	8.0
AAM-2	40–45	0	–	7.5	8.0
AC-1	40–45	0	–	8.0	8.0
AC-3	40	0.003	thiamine	6.7	n.g.
MBJ-2	45	0.003	thiamine	6.7	7.5
MJ-2	40–45	0	–	9.0	8.5
MJ-6	45	0	–	7.0	8.0
NOA-1	40–45	0	–	8.5	8.5
NOA-2	45	0.003	thiamine	6.7	8.0

[a] Data were taken for comparison from Dürre et al. 1981
[b] With adenine as a substrate that has the same redox state as hypoxanthine
n.d. = not determined
n.g. = no growth

Table 3. Requirement of bicarbonate for growth on hypoxanthine (starting pH = 7.2)

Strain	Maximal turbidity (600 nm) after supplementation with $KHCO_3$			
	none	10 mM	30 mM	100 mM
C. acidiurici 9a	–	–	0.15	0.20
C. cylindrosporum HC-1	–	–	0.53	n.d.
AAM-1	n.d.	1.23	1.44	1.02
AAM-2	–	–	0.49	0.60
AC-1	–	0.56	0.62	0.94
AC-3	–	–	–	–
MBJ-2	–	0.21	0.73	0.78
MJ-2	–	–	0.38	0.42
MJ-6	–	0.29	0.95	0.92
NOA-1	–	0.49	0.65	0.80
NOA-2	–	0.34	0.53	n.d.

– = no growth
n.d. = determined

Substrate spectra

All strains except AC-3 grew well with uric acid, xanthine, hypoxanthine, or guanine as a carbon source, respectively. AC-3 only used uric acid out of the various compounds tested. 6.8-Dihydroxypurine was a substrate for all strains except *C. acidiurici* 9a, AC-3, MBJ-2 and MJ-2. Marginal growth with 4-aminoimidazole-5-carboxamide as the substrate was observed with *C. acidiurici* 9a, *C. cylindrosporum* HC-1, AAM-1, AAM-2, AC-1, MBJ-2, MJ-2, NOA-1, and NOA-2, whereas *C. purinolyticum* WA-1 and PD-1 showed good growth with this compound as already described (Dürre et al. 1981). With the exception of WA-1, PD-1, and AAM-1, no strain was able to grow with adenine. Since only one other report described the degradation of small amounts of adenine by cell suspensions of *C. sticklandii* (Schäfer and Schwartz 1976), w tried to grow this organism in the same medium as tested with the other clostridia. However, using these conditions no growth of *C. sticklandii* with adenine was observed. *C. acidiurici* 9a, *C. cylindrosporum* HC-1, AAM-1, AAM-2, AC-1, AC-3, MBJ-2, MJ-2, MJ-6, NOA-1, and NOA-2 could not use allantoin, cytosine, formiminoglycine, fructose, glucose, glycine, glycylglycine, hippuric acid, histidine, 4-hydroxypteridin, lactate, malate, purine, pyruvate, ribose, serine, uracil, and xanthosine, respectively. Strain IMS differed considerably in growing on adenosine, formiminoglycine, glycine, glycylglycine, inosine, purine, serine, and xanthosine, thus resembling *C. purinolyticum*. The ribose moiety of adenosine was not utilized. Addition of hypoxanthine in small amounts (0.1 mM) to growth media containing amino acids, organic acids, or sugars did not show any stimulating effect. Additionally, caffeine, nicotinate, riboflavin, theobromine, theophylline, and thymine were tested as substrates for *C. acidiurici* 9a. As has been described for selenium-deficient media (Barker and Beck 1941) no growth occurred in the selenite-containing media used in this study.

Influence of selenite on growth

Selenite proved to be an essential component of the growth medium. After several transfers in selenite-deficient media with hypoxanthine as the substrate all strains failed to grow. Usually this happened after the second or third transfer. Prolonged incubation periods in the selenite-free medium even prevented outgrowth of *C. acidiurici* 9a, AAM-2, AC-1, MBJ-2, MJ-2, and NOA-1 after supplementation of the culture with 0.1 µM selenite. So only *C. cylindrosporum* HC-1, AAM-1, MJ-6, and NOA-2 were grown in selenite-containing hypoxanthine medium and the supernatants were analyzed for their respective hypoxanthine, formate, and glycine concentrations (Table 4). With xanthine as the substrate if precultured with hypoxanthine a similar dramatic effect of selenite starvation on growth could be seen only with MBJ-2 which was not able t grow. *C. cylindrosporum* HC-1, AAM-1, and MJ-6 showed a prolonged lag phase and reached lower turbidities. Comparison of the respectiv formate and glycine concentrations produced during the

Table 4. Formation of formate and glycine from various purines in selenite-supplemented (10^{-7} M) and unsupplemented media[a]

Strain	Hypoxanthine		Xanthine		Uric acid		Number of transfers (1%, v/v) in Se-deficient media resulting in growth cessation
	Formate	Glycine	Formate	Glycine	Formate	Glycine	
C. acidiurici 9a	n.g.[b]	n.g.	0.12 (0.07)[c]	0.006 (0.002)	0.09 (0.08)[c]	0.003 (0)	2
C. cylindrosporum HC-1	0.86	0.005	0.26 (0.45)	0.002 (0.002)	0.06 (n.d.)[d]	0.003 (0)	5
AAM-1	0.83	0.003	0.11 (1.03)	0.003 (0.005)	0.13 (0.20)	0.002 (0)	6
AAM-2	n.g.	n.g.	0.16 (0.16)	0.005 (0.002)	0.12 (0.13)	0.003 (0)	2
AC-1	n.g.	n.g.	0.19 (0.14)	0.006 (0.003)	0.10 (0.15)	0.002 (0)	2
AC-3	—[e]	—	—	—	0.07 (0.10)	0.003 (0)	2
MBJ-2	n.g.	n.g.	0.12 (0.13)	0.007 (0.002)	0.07 (0.10)	0.003 (0)	2
MJ-2	n.g.	n.g.	0.10 (0.08)	0.004 (0.001)	0.08 (0.08)	0.003 (0)	2
MJ-6	0.91	0.004	0.11 (1.17)	0.003 (0.002)	0.08 (n.d.)	0.003 (0)	5
NOA-1	n.g.	n.g.	0.27 (0.13)	0.007 (0.002)	0.25 (0.21)	0.003 (0)	3
NOA-2	0.86	0.002	0.10 (1.08)	0.007 (0.002)	0.16 (0.31)	0.007 (0)	5

[a] Concentrations are given in mol per mol of fermented substrate
[b] n.g. = no growth occurred after extensive selenium starvation
[c] Data in brackets represent selenium-deficient cultures
[d] n.d. = not determined
[e] — = No growth with this substrate

The strains were precultured in selenium-deficient media with hypoxanthine as substrate until only marginal growth occurred. Then washed cell suspensions were used to inoculate the different media. All media were adjusted to pH 8.0. Hydroxanthine cultures were supplemented with 30 mM $KHCO_3$ whereas xanthine and uric acid media contained 10 mM. Instead of yeast extract thiamine and biotin were added in all cases

fermentation of xanthine are given in Table 4. Strikingly, formate production in Se-deficient media was increased about tenfold with strains AAM-1, MJ-6, and NOA-2 and about twice with *C. cylindrosporum* HC-1. Under these conditions NOA-1 only produced half of the amount of formate found in a selenite-supplemented medium. In all cases glycine was formed only in trace amounts. With uric acid as the substrate all strains failed to grow after maximal 6 transfers into selenite-free medium (Table 4). Concentrations of formate, glycine, and uric acid were determined from culture supernatants after growth in the presence and absence of selenite, respectively (Table 4). Se-deficient cultures were considered those that just showed marginal growth after several transfers in selenite-free medium. Under all conditions glycine was produced only in trace amounts or not at all. No significant difference in formate formation could be observed except for strain NOA-2 that produced about twice as much formate in Se-deficient media than in selenite-supplemented cultures. Aside from the redox state of the purines the position of the hydroxyl group(s) proved to be important for growth under selenium-starvation conditions. As suggested before (Dürre and Andreesen 1982b) *C. purinolyticum* WA-1 still used 8-hydroxypurine and 6,8-dihydroxypurine in selenium-deficient cultures whereas hypoxanthine and xanthine were no longer fermented under these conditions.

Fermentation balances of various purines

Contrary to *C. acidiurici* 9a and *C. purinolyticum* HC-1 (Rakosky and Beck 1955; Rabinowitz and Barker 1956; Dürre et al. 1981) 6,8-dihydroxypurine and 4-amino-5-imidazolecarboxamide were completely fermented by *C. purinolyticum* WA-1 yielding acetate, formate, ammonia, and CO_2 as products (Table 5). No other organic acids, amino acids, ketones, or alcohols could be detected by standard gas chromatographic procedures (Vollbrecht et al. 1978). The weak growth with 4-hydroxypteridine as described before (Dürre et al. 1981) could not be substantiated by determination of the pteridine concentration. Therefore it cannot be excluded that the reported weak growth of *C. purinolyticum* on 4-hydroxypteridine was due to a contamination of this substance. As a representative of the new strains AC-1 was analyzed with respect to its growth on selenite-supplemented uric acid medium. The products formed and their respective concentrations are listed in Table 5. Glycine could only be detected in trace amounts and was therefore neglected for the calculation of the fermentation balance. AC-1 had a doubling time of 50 min under these conditions and Y_m was 15 g cells/mol of uric acid if the medium contained 0.1% (v/v) yeast extract. In a mineral medium supplemented with thiamine and biotin a Y_m of 11.4 was reached.

Sensitivity towards antibiotics, DNA base composition, and DNA/DNA-hybridization

As another criterium for strain differentiation sensitivity towards a number of antibiotics was tested for all strains. Agar diffusion tests were performed with uric acid as the substrate (Table 6).

Since all methods used so far did not allow a clear differentiation of all strains of purinolytic clostridia into distinct groups but rather showed several transitions between the three type strains, determinations of the guanine plus cytosine (G + C) content of the DNA and of the respective DNA/DNA-hybridization degrees were carried out. While all the G + C- values were found to be in the range from 27–32%, the hybridization data clearly showed that every strain could be grouped with one of the three known

Table 5. Fermentation balance of the decomposition of various purines by strains of purinolytic clostridia

Strain	Substrate and products	Concentration (mmol/100 mmol substrate)	nmol carbon	Balance of available hydr gen		O/R balance[a]	
				Available H	Available H (mmol/100 mmol substrate)	O/R value	O/R value (mmol/100 mmol substrate)
C. purinolyticum WA-1[b]	6,8-Dihydroxypurine	100	500	8	800	+6	+600
	Acetate	64	128	8	512	0	0
	Formate	25	25	2	50	+1	+25
	CO_2[c]	347	347	0	0	+2	+694
	Total		100%		70%		0.83
C. purinolyticum WA-1	4-Amino-5-imidazole-carboxamide	100	400	6	600	+4	+400
	Acetate	72	144	8	576	0	0
	Formate	77	77	2	154	+1	+72
	CO_2[c]	179	179	0	0	+2	+358
	Total		100%		121%		0.93
AC-1	Uric acid	100	500	6	600	+7	+700
	Acetate	60	120	8	480	0	0
	Formate	8	8	2	16	+1	+8
	CO_2[c]	372	372	0	0	+2	+744
	Total		100%		83%	4	1.07

[a] Calculation was performed as described before (Dürre and Andreesen 1982c)
[b] Data were obtained from samples taken at the beginning and at the end of the exponential growth phase. NH_3 was not determined
[c] Calculated from carbon recovery

type strains (Table 7). According to Johnson (1973) strains with DNA homologies of 70% and more were considered to be the same species. However, some transitions could be observed (e.g. MBJ-2 to *C. cylindrosporum* HC-1, NOA-1 to *C. acidiurici* 9a, PD-1 to *C. acidiurici* 9a).

Enzymatic investigations

The striking feature of strain AC-3 to grow only with uric acid as a substrate led to the question whether it had a functioning xanthine dehydrogenase, the key enzyme for purine interconversion, or rather fermented uric acid via the pyrimidine pathway (Dürre and Andreesen 1982b). Crude extracts exhibited a specific activity of xanthine dehydrogenase of 2 U/mg protein that was within the range usually observed in strains of purinolytic clostridia (Table 8). The enzyme could only be detected in the supernatant after breaking up the cells by a French pressure treatment and centrifugation at 40,000 $\times g$. The enzyme lost about 20% of its activity after storage for 3 days at 4° C and had an optimal pH-value of 8.9 for the reaction. The apparent K_m for xanthine was 0.5 mM. Xanthine dehydrogenase of strain AC-3 had a high substrate specificity. In contrast to enzymes from *C. acidiurici* 9a (Wagner 1980), *C. cylindrosporum* HC-1 (Bradshaw and Barker 1960), and *C. purinolyticum* WA-1 (Dürre et al. 1981) the xanthine dehydrogenase of AC-3 in crude extracts did not react at all with adenine, hypoxanthine, or purine, respectively. Adenine inhibited the reaction with xanthine by 44% if added in equimolar concentrations. Comparing the substrate specificity of the xanthine dehydrogenase in extracts of the other strains, no general trend could be observed what might explain their growth behavior.

The strains were grown in the presence of 0.1% yeast extract under three conditions known to affect differently formate dehydrogenase activity (Wagner and Andreesen 1977): i) no supplementation with selenium, molybdenum, or tungsten; ii) addition of selenite and molybdate; iii) addition of selenite and tungstate. Although it had been suggested that the trace elements molybdenum and tungsten — both in combination with selenium — exert a species specific influence on formate dehydrogenase activity, this could bot be substantiated to be a taxonomic marker after examination of other strains of purinolytic clostridia (Table 8). At least, the positive action of tungsten besides selenium on formate dehydrogenase could be shown for many strains, and there was no or no pronounced negative influence of tungstate on xanthine dehydrogenase.

Discussion

Cell and spore morphology, features that originally led to the differentiation between *Clostridium acidiurici* and *C. cylindrosporum*, do not allow an unequivocal grouping of all purinolytic clostridia known so far. While it is easy to identify strains of *C. purinolyticum* by their exclusive ability

Table 6. Antibiograms of purinolytic clostridia[a]

Antibiotics	Strain												
	C. acidiurici 9a	*C. cylindrosporum* HC-1	*C. purinolyticum* WA-1	AAM-1	AAM2	AC-1	AC-3	MBJ-2	MJ-2	MJ-6	NOA-1	NOA-2	PD-1
Ampicillin (10 µg)	+	+	+	+	+	+	+	+	+	+	−	+	+
Bacitracin (10 µg)	+	+	+	+	−	+	+	+	+	+	+	+	+
Chloramphenicol (30 µg)	+	+	+	0	+	+	+	+	+	+	+	+	+
Chlortetracycline (30 µg)	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythromycin (15 µg)	+	+	+	+	+	+	+	+	+	+	+	+	+
Kanamycin (1 mg)	+	+	0	+	+	−	−	+	−	+	−	+	+
Lincomycin (2 µg)	−	0	−	+	−	0	−	−	+	0	+	−	−
Nitrofurantoin (100 µg)	+	+	+	+	+	+	−	+	+	+	0	+	+
Oleandomycin (15 µg)	−	0	−	+	+	+	−	+	+	+	+	−	−
Oxytetracycline (15 µg)	+	+	+	+	+	−	+	+	0	+	−	+	+
Penicillin G (2 I.E.)	−	+	−	+	−	0	−	−	+	+	+	−	+
Polymyxin B (300 I.E.)	−	−	−	−	−	−	−	−	−	−	−	−	−
Streptomycin (10 µg)	−	−	−	−	−	−	−	−	−	−	−	−	−
Sulfisoxazole (2 mg)	−	+	−	+	−	−	−	+	0	+	−	−	−
Tetracycline (30 µg)	+	+	+	+	+	−	−	+	0	+	−	+	+
Vancomycin (5 µg)	0	+	+	+	+	−	−	0	0	+	−	+	+

[a] Sensitivity was classified according to three categories: − = resistant (no inhibition zones), 0 = moderate sensitive (diameter of inhibition zones ≤ 14 mm), and + = sensitive (diameter of inhibition zones > 14 mm)

Table 7. Mol percent guanine plus cytosine and degrees of DNA homology among purinolytic clostridia

Strain	G + C content (mol%)	% DNA homology with		
		C. acidiurici 9a	*C. cylindrosporum* HC-1	*C. purinolyticum* WA-1
C. acidiurici 9a	27.8[b]	100[a]		
C. cylindrosporum HC-1	27.9[a]	30.4[a]	100[a]	
C. purinolyticum WA-1	29.0[a]	20.7[a]	33.7[a]	100[a]
AAM-1	29.3[a]	22.0[a]	12.3[a]	67.1
AAM-2	30.4	24.4	73.3	56.6
AC-1	27.7	84.9	41.7	30.1
AC-3	27.0	88.2	40.1	44.3
IMS	32.1	n.d.	n.d.	73.2
MBJ-2	30.2	108.2	59.3	9.8
MJ-2	30.4	87.6	40.1	21.1
MJ-6.	29.3	42.2	93.6	45.4
NOA-1	27.2	64.9	108.9	32.8
NOA-2	29.4	45.3	74.6	51.4
PD-1	29.3[a]	55.9[a]	42.3[a]	82.9[a]

[a] Data were taken for comparison from Dürre et al. (1981) n.d. = not determined

Table 8
Enzyme levels after growth with different trace element supplementations

Strain	Formate dehydrogenase (U per mg of protein)	Element necessary for highest activity besides Se	Xanthine dehydrogenase (U per mg of protein)
C. acidiurici 9a[a]	11.3	W > Mo	4.4
C. cylindrosporum HC-1[a]	8.0	Mo > W	3.7
C. purinolyticum WA-1[b]	1.8	Mo > W	1.6
AAM-1	0.6	Mo > W	1.9
AAM-2	3.9	Mo = W	1.8
AC-1	5.7	W > Mo	1.7
AC-3	4.1	W = Mo	2.0
MBJ-2	5.6	Mo = W	7.5
MJ-2	1.7	W > Mo	1.2
MJ-6	3.8	Mo > W	1.3
NOA-1	7.3	W > M	1.6
NOA-2	4.7	W > Mo	1.4

[a] Data were taken from Wagner and Andreesen (1977, 1979)
[b] Data were taken from Dürre et al. (1981)
Both enzyme activities were measured in 0.1 M potassium phosphate buffer, pH 7.9, using methyl viologen as electron acceptor. The cells were grown in the presence of selenite and either tungstate or molybdate, 10^{-7} M each

to ferment adenine and/or glycine (Dürre et al. 1981) substrate spectra of the other clostridia do not reveal clear differences. Strain AC-3 might be restricted to uric acid for growth by its unusually substrate-specific xanthine dehydrogenase in addition to a defective or missing transport system, at least for xanthine. Although the three species were reported to vary in their Gram reaction, being Gram-positive in case of *C. purinolyticum*, sometimes Gram-positive in case of *C. acidiurici*, or Gram-negative in case of *C. cylindrosporum* (Barker and Beck 1942; Dürre et al. 1981), this pattern could be shifted to a Gram-positive reaction by using actively growing cell material. Thus, only one strain (IMS) reacted in most cases Gram-negative. In addition, nutritional requirements, product formation, and sensitivity towards antibiotics are not sufficient for differentiation. For each semblence of a correlation, there are exceptions. For example, AAM-1, AC-3, MBJ-2, and NOA-2 all show almost identical vitamin requirements and pH optima. However, they produce quite different amounts of formate and differ considerably in their sensitivity towards antibiotics. Furthermore, it became clear from this study that media variations can cause dramatic shifts in fermentation products (e.g. formate concentrations). The 10-fold increased amount of formate from fermentation of xanthine in selenite-deficient cultures of AAM-1, MJ-6, and NOA-2 could be explained by assuming formate dehydrogenase to be a selenoenzyme as has been proposed for *C. acidiurici* 9a and *C. cylindrosporum* HC-1 (Wagner and Andreesen 1977). Under these conditions not enough active enzyme would be present to oxidize formate to CO_2 resulting in higher concentrations of this compound in the medium. Selenite-supplemented cultures of all strains never produced significant amounts of glycine. This makes sense for all three type strains contain a glycine reductase (Dürre and Andreesen 1983), a selenoenzyme yielding ATP from the reduction of glycine to acetate (Stadtman 1980). Interestingly, in our hands even in selenium-deficient cultures only trace amounts of glycine could be detected once the organisms had be grown before in the presence of selenite. We do not yet have an explanation for this phenomenon. The relatively high Y_m of strain AC-1 as also observed for *C. purinolyticum* (Dürre et al. 1981; Dürre and Andreesen 1982c) might indicate additional energy generation by a glycine reductase.

Strains grouped with *C. acidiurici* according to Champion and Rabinowitz (1977) generally showed a somewhat faster response to selenium deficiency. The observed more pronounced dependence on selenium during growth on hypoxanthine compared to xanthine and uric acid might be explained by the necessity of xanthine dehydrogenase for their metabolism (Dürre and Andreesen 1982a, b, 1983). Selenium is required for xanthine dehydrogenase activity and part of the enzyme (Wagner and Andreesen 1979; Wagner 1980). Regarding formate dehydrogenase activity, a differentiation on the basis of specific trace element requirements — as shown for the type species (Wagner and Andreesen 1977) — proved to be no consistent taxonomic marker.

A requirement for thiamine as observed for some strains might be a consequence of their specialized metabolism. Biosynthesis of purines and of the pyrimidine ring of thiamine proceeds via common steps up to 4-aminoimidazole ribonucleotide (Newell and Tucker 1968). It seems logical that bacteria specialized on purine fermentation lost their capacity to synthesize purines de novo. Therefore, they do require a purine as supplement growing on glycine (Dürre and Andreesen 1982c, 1983). Some strains might be able to form 4-aminoimidazole ribonucleotide from 4-aminoimidazole, an intermediate of purine breakdown (Dürre and Andreesen 1982a). Those strains, therefore, would not suffer from their metabolic lesion.

From the 13 strains tested not even two proved to have an identical antibiogram revealing this method to be useless in the classification of the purinolytic clostridia. Only polymyxin B and streptomycin were completely inactive against all strains. The composition of the DNA (G + C values) also did not help in differentiation. All strains were in the range of 27 – 32% G + C, values typical for clostridia. Deviations compared to data reported earlier for *C. acidiurici* and *C. cylindrosporum* (Tonomura et al. 1965) might be due to different periods of time before cell harvest (Garvie 1979).

On the other hand the DNA/DNA-hybridization data clearly show that *C. acidiurici* 9a and *C. cylindrosporum* HC-1 are two distinct species. Adding *C. purinolyticum* to this list of known obligately purinolytic clostridia all strains isolated so far can be grouped together with one of the three type strains by means of the degree of DNA homology. A dendrogram proposed on the basis of these data is shown in Fig. 1. With three exceptions this phylogenetic tree is identical to the scheme proposed by Champion and Rabinowitz (1977). They compared physical characteristics, immunological properties, and amino acid compositions of the respective ferredoxins as well as immunodiffusion results with formyltetrahydrofolate synthetase preparations. From these data they already concluded that strain AAM-1 behaved rather atypically which later found its explanation by the fact that this strain is a subspecies of *C. purinolyticum* (Dürre et al. 1981). Thus, the only real difference is that we classified AAM-2 and NOA-1 as strains of *C. cylindrosporum* rather than *C. acidiurici*. However, NOA-1 also showed a rather high degree of DNA homology with *C. acidiurici* (as well as AAM-2 with *C. purinolyticum*) indicating that they might be transition forms. We suggest the new classification of these two strains considering the DNA homology data to be more convincingly, since DNA reassociation experiments compare the whole genetic potential of the purinolytic clostridia rather than two proteins. This technique is thought to be the method of choice for determining biological relatedness at the species level (Bradley 1980). However, both approaches definitely prove that *C. acidiurici* and *C. cylindrosporum* are distinct species. Neither Bergey's Manual (Smith and Hobbs 1974) nor the Approved Lists of Bacterial Names (Skerman et al. 1980) list *C. cylindrosporum* as a proper species. Support for listing *C. cylindrosporum* as own species is given by Woese (cited as personal communication in Tanner et al. 1982) on the basis of clear differences to *C. acidiurici* in the respective 16S rRNA catalogs. *C. purinolyticum* exhibits low S_{AB}-values compared to both species (R. Tanner, personal communication). Thus, an unequivocal differentiation between all these clostridia is possible, though only by laborious and time-consuming methods. It is not yet known whether the three type strains are the only obligately purinolytic clostridial species. All one can say is that all strains known so far belong to one of these species.

It is somewhat surprising that the high degree of phenotypic similarity of these bacteria is not reflected in genotypic relatedness. So the question comes up as whether

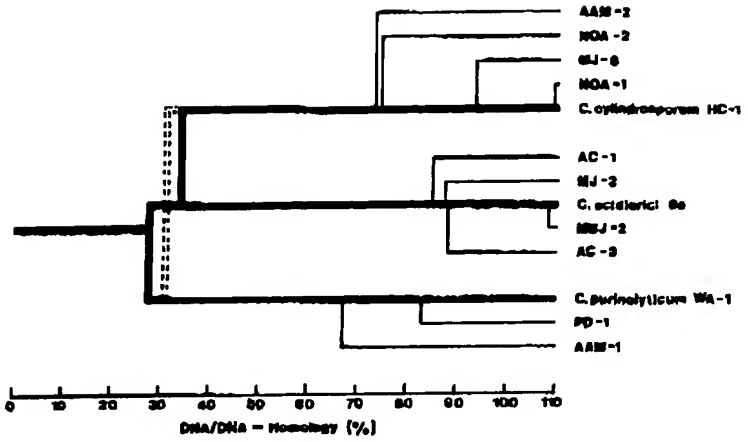

Fig. 1
Phylogenetic relationship of obligately purinolytic clostridia on the basis of DNA homology. With the exception of the three type strains (*dotted lines*) only the highest values were used

this is a case of convergent or divergent evolution. Champion and Rabinowitz (1977) opted for divergence because of the many physiological similarities. Furthermore, they felt that a strong argument against a convergence was the fact that other anaerobic bacteria developed different pathways of purine degradation (Whiteley 1952). Recent studies, however, indicate that at least *Peptococcus aerogenes* degrades purines via the clostridial imidazole pathway in selenite-supplemented media (Spahr 1982). Also, a purine fermentation via pyrimidine derivatives as suggested for *P. aerogenes* (Whiteley 1952; Vogels and van der Drift 1976) has been detected in *C. purinolyticum* (Dürre and Andreesen 1982b). However, we feel that the facts are too scarce to give either possibility a preference. Hopefully, comparative cataloging of 16S ribosomal RNA will allow further evolutionary insights.

Some of the physiological characteristics of these clostridia deserve attention and might point to an ecological niche that suits such organisms. Increasing the pH up to 8.5 resulted in faster growth. Doubling times of 50 min as for AC-1 are remarkably quick for anaerobic bacteria. The optimal growth temperatures of 40–45°C as determined for most strains correspond well to body temperatures of 41–44°C of birds. Thus, the ubiquitous dissemination of these microorganisms (Barker and Beck 1942; Champion and Rabinowitz 1977; Emtsev and Babaitseva 1978) could be easily explained. Already Barker and Beck (1942) isolated one of their *C. acidiurici*-strains from fecal material of the yellowshafted flicker. The avian gut also provides alkaline conditions corresponding to the high pH optima found in this study.

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